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Lignicolous freshwater ascomycota from Thailand: Phylogenetic and morphological characterisation of two new freshwater fungi: *Tingoldiago hydei* sp. nov. and *T. clavata* sp. nov. from Eastern Thailand

Li Xu[1], Dan-Feng Bao[2,3,4], Zong-Long Luo[2], Xi-Jun Su[2], Hong-Wei Shen[2,3], Hong-Yan Su[2]

1 *College of Basic Medicine, Dali University, Dali 671003, Yunnan, China* **2** *College of Agriculture & Biological Sciences, Dali University, Dali 671003, Yunnan, China* **3** *Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand* **4** *Department of Entomology & Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*

Corresponding author: Hong-Yan Su (suhongyan16@163.com)

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Abstract

Lignicolous freshwater fungi represent one of the largest groups of Ascomycota. This taxonomically highly diverse group plays an important role in nutrient and carbon cycling, biological diversity and ecosystem functioning. The diversity of lignicolous freshwater fungi along a north-south latitudinal gradient is currently being studied in Asia. In this paper, we introduce two novel freshwater taxa viz. *Tingoldiago hydei* **sp. nov.** and *T. clavata* **sp. nov.** which were collected from freshwater substrates in Eastern Thailand. Morphological comparison based on the size of ascomata, asci and ascospores, as well as multi-gene phylogenetic analyses based on LSU, SSU, ITS and TEF1-α DNA sequences, supports their placement in *Tingoldiago* (Lentitheciaceae). Descriptions and illustrations of these two new species are provided.

Keywords

2 new species, Lentitheciaceae, Freshwater fungi, phylogeny, taxonomy

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Introduction

Freshwater fungi are those which the whole or part of their life cycle is found in a freshwater habitat (Thomas 1996, Wong et al. 1998) and they are an evolutionary important group (Vijaykrishna et al. 2006). The members of freshwater fungi can be saprobes, parasites, endophytes and mutualistic taxa (Vijaykrishna et al. 2005, Zhang et al. 2008, Swe et al. 2009, Jones et al. 2014, Huang et al. 2018). There is a wide range of organisms that can be freshwater fungi hosts, such as wood, plants, alga, foams, fish etc. (Sparrow 1960, Ellis and Ellis 1985, Jones et al. 2014). However, a lot of studies on freshwater fungi have focused on lignicolous freshwater fungi (Tsui et al. 2000, Cai et al. 2002, Luo et al. 2004, 2018, Jones et al. 2014, Hyde et al. 2016, Yang et al. 2017), which were defined as those fungi that grow on submerged woody debris in freshwater streams, ponds, lakes and tree hollows (Hyde et al. 2016). They also grow on submerged wood in peat swamps and dams (Pinnoi et al. 2006, Pinruan et al. 2007, 2014, Hu et al. 2010). Lignicolous freshwater fungi are a diverse group comprising species from different phyla (Aphelidiomycota, Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Monoblepharomycota, Mortierellomycota and Rozellomycota) (Shearer et al. 2007, Kagami et al. 2012, Zhang et al. 2012, Jones et al. 2014, Wijayawardene et al. 2018). The dominant groups of lignicolous freshwater fungi are Dothideomycetes and Sordarialmycets (Jones et al. 2014, Hyde et al. 2016, Wijayawardene et al. 2017, 2018).

We are studying the diversity of lignicolous freshwater fungi in Thailand, in order to establish the phylogenetic relationships of lignicolous freshwater fungi, understanding the natural classification of this group and contributing to the biogeographical diversity of fungi (Hyde et al. 2016). The study on freshwater fungi in Thailand was first investigated by Tubaki et al. (1983) and they reported 40 freshwater fungal species from foam. Subsequently, mycologists started to study lignicolous freshwater fungi in Thailand and several taxa have been reported (Sivichai et al. 1998, 1999, 2000, 2002, 2010, Jones et al. 1999, Marvanová et al. 2000, Hu et al. 2010, Zhang et al. 2013, Luo et al. 2015, 2016, Bao et al. 2018).

Lentitheciaceae was introduced by Zhang et al. (2012) to accommodate *Massarina*-like species in the order Pleosporales. Presently, 13 genera are accepted in this family (Dayarathne et al. 2018, Hyde et al. 2018). Species in this family are widely distributed in the world (China, Egypt, Hungary, Italy, Japan, Russia, Saudi, Thailand, UK, Uzbekistan) and are commonly saprobic on stems and twigs of herbaceous and woody plants in terrestrial or aquatic habitats (Wanasinghe et al. 2014, 2018, Knapp et al. 2015, Wijayawardene et al. 2015, Luo et al. 2016, Tibpromma et al. 2017, Hyde et al. 2018). The genus *Tingoldiago* was established by Hirayama et al. (2010) with a single species *Tingoldiago graminicola* K. Hiray. & Kaz. Tanak, this species being originally treated as *Massarina ingoldiana*. Later, Hirayama et al. (2010) re-assessed the phylogeny of *Massarina ingoldiana* and introduced two new genera *Tingoldiago and Lindgomyces* to accommodate *Massarina ingoldiana sensu lato*, based on phylogenetic analyses. Currently, only one species is accepted in this genus.

In this paper, we introduce two new freshwater species of *Tingoldiago* (Lentitheciaceae), based on morpho-molecular studies. Detailed descriptions and illustrations of these two new species are provided.

Materials and methods

Collection, Isolation and morphological studies

Submerged decaying wood samples were collected from That Phanom, Nakhon Phanom, Thailand and brought to the laboratory in plastic bags. The samples were incubated in plastic boxes lined with moistened tissue paper at room temperature for one week. Specimen observations and morphological studies were conducted, following the protocols provided by Luo et al. (2018).

Pure cultures were obtained by single spore isolation followed by Chomnunti et al. (2014). Germinating ascospores were transferred aseptically to potato dextrose agar (PDA) plates and grown at 16–25 °C in daylight. Colony colour and other characters were observed and measured after three weeks. The specimens were deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Living cultures are deposited in the Culture Collection of Mae Fah Luang University (MFLUCC). Facesoffungi numbers and Index Fungorum numbers were obtained, following Jayasiri et al. (2015) and Index Fungorum (2019). New species have been established as recommended by Jeewon and Hyde (2016).

DNA extraction, PCR amplification and sequencing

Fungal mycelium was scraped from the surface of colonies grown on a PDA plate or MEA plate at 25 °C for 4 weeks, transferred into a 1.5 ml centrifuge tube and ground using liquid nitrogen. The EZ geneTM fungal gDNA kit (GD2416) was used to extract DNA from the ground mycelium according to the manufacturer's instructions. The gene regions of the large subunit of the nuclear ribosomal DNA (LSU), the internal transcribed spacers (ITS), the small subunit of the nuclear ribosomal DNA (SSU) and the translation elongation factor (TEF1-α) RNA were amplified using the primer pairs LR0R/LR7 (Vilgalys and Hester 1990), ITS5/ITS4, NS1/ NS4 (White et al. 1990) and 983F/2218R (Liu et al. 1999), respectively. The amplification reactions were performed in 25 µl of PCR mixtures containing 9.5 µl ddH_2O, 12.5 µl 2× PCR MasterMix (Tsingke Co., China), 1 µl DNA sample and 1µl of each primer. The PCR thermal cycle programme for LSU, ITS, SSU and TEF1-α amplification were as follows: 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 50 seconds, elongation at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes and finally kept at 4 °C. PCR amplification was confirmed on 1% agarose electrophoresis gels

stained with ethidium bromide. PCR products were sequenced using the same set of primers used in PCR in Beijing Tsingke Biological Engineering Technology and Services Co. Ltd. (Beijing, P.R. China).

Sequencing and sequence alignment

The sequence was assembled by using BioEdit and sequences with high similarity indices were determined from a BLAST search to find the closest matches with taxa in Lentitheciaceae and from recently published data (Dayarathne et al. 2018). All consensus sequences and the reference sequences were aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), then checked visually and manually optimised using BioEdit v.7.0.9 (Hall 1999). Ambiguous regions were excluded from the analyses and gaps were treated as missing data. The phylogeny website tool "ALTER" (Glez-Peña et al. 2010) was used to convert the alignment fasta file to Phylip format for RAxML analysis and Clustalx BETA and PAUP 4.0 were used to convert the alignment fasta file to a Nexus file for Bayesian analysis. Phylogenetic analyses were obtained from Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian analysis.

Phylogenetic analyses

Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2006, Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010) using GTR+ I + G model of evolution which was estimated by MrModeltest 2.2 (Nylander et al. 2008). Maximum likelihood bootstrap values (ML), equal to or greater than 75%, are given above each node (Figure 1).

MP analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BS) analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa (Hillis and Bull 1993).

The Bayesian analysis was performed with MrBayes v3.2 (Ronquist et al. 2012), with the best-fit model of sequence evolution estimated with MrModeltest 2.2 (Nylander et al. 2008) to evaluate posterior probabilities (PP) (Rannala and Yang 1996, Zhaxybayeva and Gogarten 2002) by Markov Chain Monte Carlo (MCMC) sampling. Six simultaneous Markov chains were run for 10,000,000 generations, trees were sampled every 1000th generation and 1,0000 trees were obtained. Based on the tracer analysis, the first 1,000 trees representing 10% were discarded as the burn-in phase in the analysis. The remaining trees were used to calculate posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01).

Table 1. Taxa used in this study and their GenBank accession numbers, the newly generated sequences are indicated wirh * and the type strains are indicated in bold.

Taxa	strain	GenBank accession number			
		LSU	SSU	ITS	TEF1
Bambusicola bambusae	**MFLUCC 11–0614**	**JX442035**	**JX442039**	**NR121546**	**KP761722**
B. irregulispora	**MFLUCC 11–0437**	**JX442036**	**JX442040**	**NR121547**	**KP761723**
B. massarinia	**MFLUCC 11–0389**	**JX442037**	**JX442041**	**NR121548**	–
Bimuria novaezelandiae	AFTOL ID931	–	–	–	DQ471087
Byssothecium circinans	**CBS67592**	**GU205217**	**GU205235**	–	**GU349061**
Corynespora cassiicola	CBS100822	GU301808	GU296144	–	GU349052
C. smithii	CABI5649b	GU323201	–	–	GU349018
Dacampia engeliana	**72868**	**KT383791**	–	–	–
D. hookeri	**74269**	**KT383793**	–	–	–
D. hookeri	**81840**	**KT383795**	–	–	–
Darksidea alpha	**CBS 135650**	**KP184019**	**KP184049**	**NR137619**	**KP184166**
D. beta	**CBS 135637**	**KP184023**	**KP184049**	**NR137957**	**KP184189**
D. delta	**CBS 135638**	–	–	**NR137075**	–
D. epsilon	**CBS 135658**	**KP184029**	**KP184070**	**NR137959**	**KP184186**
D. gamma	**CBS 135634**	**KP184031**	**KP184073**	**NR137587**	**KP184188**
D. zeta	**CBS 135640**	**KP184013**	**KP184071**	**NR137958**	**KP184191**
Falciformispora lignatilis	BCC 21117	GU371826	GU371834	KF432942	GU371819
F. lignatilis	BCC 21118	GU371827	GU371835	KF432943	GU371820
Halobyssothecium obiones	27AV2385	–	–	KX263864	–
H. obiones	MFLUCC 15–0381	MH376744	MH376745	MH377060	MH376746
Helicascus nypae	**BCC36752**	**GU479789**	**GU479755**	–	**GU479855**
Kalmusia scabrispora	KT2202	AB524594	AB524453	–	AB539107
Karstenula rhodostoma	CBS69094	GU301821	GU296154	–	GU349067
Katumotoa bambusicola	**KT1517a**	**AB524595**	**AB524454**	**LC014560**	**AB539108**
Keissleriella breviasca	KT649	AB807588	AB797298	–	AB808567
K. culmifida	KT2642	AB807592	AB797302	LC014562	–
K. gloeospora	KT829	AB807589	AB797299	LC014563	–
K. poagena	CBS136767	KJ869170	–	KJ869112	–
K. quadriseptata	KT2292	AB807593	AB797303	AB811456	AB808572
K. taminensis	KT571	AB807595	AB797305	LC014564	AB808574
K. trichophoricola	CBS 136770	KJ869171	–	KJ869113	–
Lentithecium clionina	KT1149A	AB807540	AB797250	LC014566	AB808515
L. fluviatile	**CBS 123090**	**FJ795450**	**FJ795492**	–	–
L. pseudoclioninum	KT1111	AB807544	AB797254	AB809632	AB808520
Massarina cisti	**CBS 266 62**	**FJ795447**	**FJ795490**	**LC014568**	**AB808514**
M. eburnea	CBS 473 64	GU301840	GU296170	–	GU349040
Montagnula opulenta	AFTOLID1734	DQ678086	AF164370	–	–
Morosphaeria ramunculicola	**JK5304B**	**GU479794**	**GU479760**	–	–
Murilentithecium clematidis	IT1078	KM408758	KM408760	KM408756	–
M. clematidis	MFLUCC 14–0562	KM408759	KM408761	KM408757	KM454445
Neoophiosphaerella sasicola	KT1706	AB524599	AB524458	LC014577	AB539111
Palmiascoma gregariascomum	MFLUCC 11–0175	KP744495	KP753958	KP744452	–
Paraconiothyrium brasiliense	CBS100299	JX496124	AY642523	JX496011	–
Paraphaeosphaeria michotii	MFLUCC 13–0349	KJ939282	KJ939285	KJ939279	–
P. minitans	CBS122788	EU754173	EU754074	–	GU349083
Phaeodothis winteri	CBS18258	–	GU296183	–	–
Phragmocamarosporium platani	MFLUCC 14–1191	KP842915	KP842918	–	–

Taxa	strain	GenBank accession number			
		LSU	SSU	ITS	TEF1
Pleurophoma ossicola	CBS139905	KR476769	–	KR476736	–
P. ossicola	**CPC24985**	**KR476770**	**–**	**NR137992**	**–**
Pleurophoma pleurospora	CBS130329	JF740327	–	–	–
Poaceascoma aquaticum	**MFLUCC 14–0048**	**KT324690**	**KT324691**	**–**	**–**
P. halophila	**MFLUCC 15–0949**	**MF615399**	**MF615400**	**–**	**–**
P. helicoides	**MFLUCC 11–0136**	**KP998462**	**KP998463**	**KP998459**	**KP998461**
Pseudomurilentithecium camporesii	**MDLUCC 14-1118**	**MN638846**	**MN638850**	**MN638861**	**–**
Setoseptoria arundinacea	KT600	AB807575	AB797285	LC014595	AB808551
S. magniarundinacea	KT1174	AB807576	AB797286	LC014596	AB808552
S. phragmitis	**CBS 114802**	**KF251752**	**–**	**KF251249**	**–**
S. scirpi	**MFUCC 14–0811**	**KY770982**	**KY770980**	**MF939637**	**KY770981**
Stagonospora macropycnidia	CBS 114202	GU301873	GU296198	–	GU349026
Tingoldiago graminicola	KH155	AB521745	AB521728	LC014599	AB808562
T. graminicola	KH68	AB521743	AB521726	LC014598	AB808561
T. graminicola	KT891	AB521744	AB521727	–	AB808563
****T. hydei***	**MFLUCC 19-0499**	**MN857177**	**–**	**MN857181**	**–**
****T. clavata***	**MFLUCC 19-0496**	**MN857178**	**MN857186**	**MN857182**	**–**
**T. clavata*	MFLUCC 19-0498	MN857179	MN857187	MN857183	–
**T. clavata*	MFLUCC 19-0495	MN857180	MN857188	MN857184	–
Towyspora aestuari	**MFLUCC 15–1274**	**KU248852**	**KU248853**	**NR148095**	**–**
Trematosphaeria pertusa	**CBS 122368**	**FJ201990**	**FJ201991**	**NR132040**	**KF015701**
Trematosphaeria pertusa	CBS 122371	GU301876	GU348999	KF015669	KF015702

The phylograms were visualised in FigTree 1.4.2 (Rambaut 2014) and made in Adobe Illustrator CS5 (Adobe Systems Inc., USA). All newly generated sequences of this study have been submitted in GenBank.

Results

Phylogenetic analyses

The aligned sequence matrix comprises LSU, SSU, ITS and TEF1-α sequence data for 69 taxa, with *Corynespora smithii* and *Corynespora cassiicola* as out-group taxa. The dataset comprises 3334 characters after alignment including gaps (LSU: 1–897; SSU: 898–1920; ITS: 1921–2522; TEF1-α: 2523–3479). The topologies of RAxML, MP and Bayesian are similar and the bootstrap support values for Maximum Likelihood (ML), Maximum Parsimony (MP) higher than 75% and Bayesian posterior probabilities (PP) greater than 0.95 are given above the nodes. Maximum parsimony analyses indicated that 2,442 characters were constant, 232 variable characters parsimony uninformative and 805 characters are parsimony-informative. The RAxML analysis of the combined dataset yielded the best scoring tree (Figure 1) with a final ML optimisation likelihood value of -21568.713178. The matrix had 1322 distinct alignment patterns,

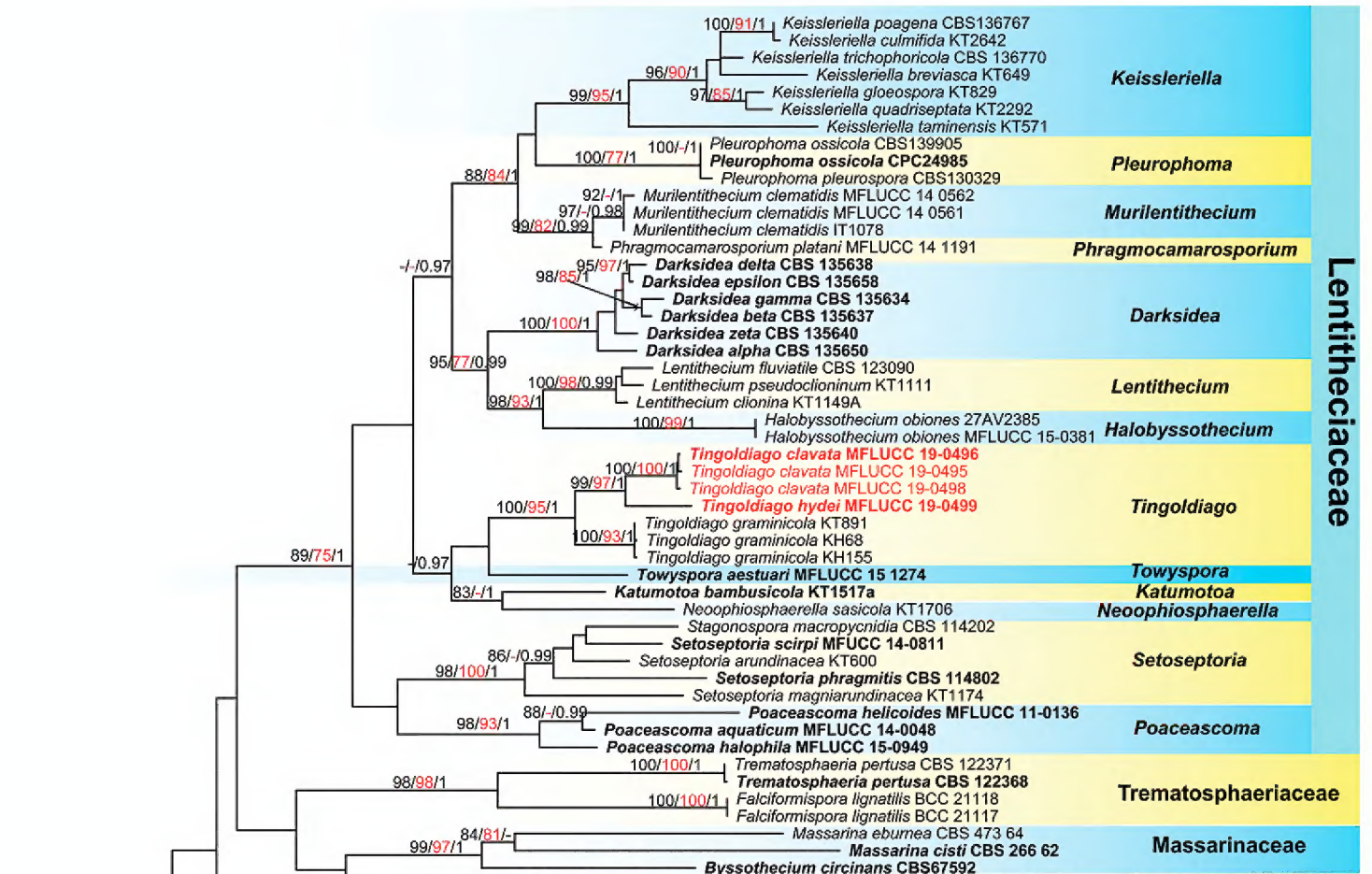

Figure 1. Phylogenetic tree based on RAxML analyses of combined LSU, SSU, ITS and TEF1-α sequence data. Bootstrap support values for maximum likelihood (ML, black) and maximum parsimony (MP, red) higher than 75% and Bayesian posterior probabilities (PP, black) greater than 0.95 are indicated above the nodes as MP / ML /PP. The ex-type strains are in bold and the newly obtained isolates are in red. The tree is rooted at *Corynespora smithii* (CABI5649b) and *Corynespora cassiicola* (CBS100822).

with 30.89% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.238228, C = 0.248262, G = 0.272670, T = 0.240839; substitution rates AC = 1.161111, AG = 2.490274, AT = 1.596115, CG = 1.194931, CT = 7.261814, GT = 1.000000; gamma distribution shape parameter α = 0.183824.

The novel species *Tingoldiago hydei* and *T. clavata*, introduced in this paper, are supported by multi-phylogenetic analyses. Four newly generated strains clustered together within *Tingoldiago* with strong statistical support (100 ML/95 MP/1.00 PP, Figure. 1). Three strains of *T. clavata* clustered together and sister to *T. hydei* with strong bootstrap support (99 ML/97 MP/1 PP, Figure 1).

Taxonomy

Tingoldiago hydei **D.F. Bao, Z.L. Luo & H.Y. Su, sp. nov.**
Index Fungorum No: IF557047
Facesoffungi No: FoF07082
Figure 2

Etymology. Referring to Kevin D. Hyde for his contributions in fungal taxonomy.

Holotype. Thailand, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B-126 (MFLU 19–2842, holotype), ex-type living culture, MFLUCC 19–0499.

Description. *Saprobic* on submerged decaying wood. ***Sexual morph:*** *Ascomata* 180–280 × 330–470 μm ($\overline{x}$ = 400 × 420 μm, n = 10), immersed to semi-immersed, erumpentia, gregarious, scattered, depressed globose to conical with a flattened base, dark brown to black, as dark spots on host surface. *Ostioles* central, papillate, short, crest-like, dark brown. *Peridium* 33.5–50 μm wide, comprising 4–6 layers, brown to dark brown cells of *textura anngularis*. *Hamathecium* comprising 2–2.5 μm (n = 30) wide, numerous, branched, septate, hyaline, cellular pseudoparaphyses. *Asci* 95–164 × 18–22 μm ($\overline{x}$ = 129 × 20 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical-clavate, rounded at apex, with a short pedicellate. *Ascospores* 37.5–42 × 7.5–9 μm ($\overline{x}$ = 40 × 8 μm, n = 30), overlapping, 2–3-seriate, clavate with round ends, straight, uniseptate, deeply constricted at septum, with broad and short upper cells 17.5–20 × 7–8.7 μm ($\overline{x}$ = 18.7 × 7.9 μm, n = 30), narrow and long lower cells 20.6–23.3 × 5.9–7.4 μm ($\overline{x}$ = 21.9 × 6.7 μm, n = 30), tapering towards the end, with short appendages at the septum, hyaline, guttulate, smooth, surrounded by a fusiform gelatinous sheath. ***Asexual morph:*** Undetermined.

Culture characteristics. Ascospores germinating on PDA within 24 hours. Colonies on MEA effuse, greyish-white to dark brown from above and below, reaching 3–4 cm diameter within 30 days at room temperature under natural light, composed of subhyaline to pale brown, septate, smooth hyphae.

Notes. Phylogenetic analysis showed that *Tingoldiago hydei* is related to *T. clavata*; however, they are in different lineages with significant support (99 ML/97 MP/1.00 PP, Figure 1). *Tingoldiago hydei* resembles *T. clavata* in having bitunicate, cylindrical-clavate asci and clavate, hyaline, uniseptate, ascospores with broad and short upper cells, narrow and long lower cells, tapering towards the end, surrounded by a gelatinous sheath. However, *Tingoldiago hydei* can be distinguished from *T. clavata* in having longer and narrower asci (95–164 × 18–22 vs. 110–148 × 20–27 μm) and smaller ascospores (37.5–42 × 7.5–9 vs. 48–51 × 7.5–8.5 μm). Moreover, ascospores of *T. clavata* have longer appendages at the septum, while the appendages of *T. hydei* are much shorter than *T. hydei*.

Tingoldiago clavata is similar to the type species, *T. raminicola* in having immersed to semi-immersed, depressed globose to conical ascomata with flattened base, bitunicate, fissitunicate, cylindrical-clavate asci and clavate, straight, uniseptate ascospores.

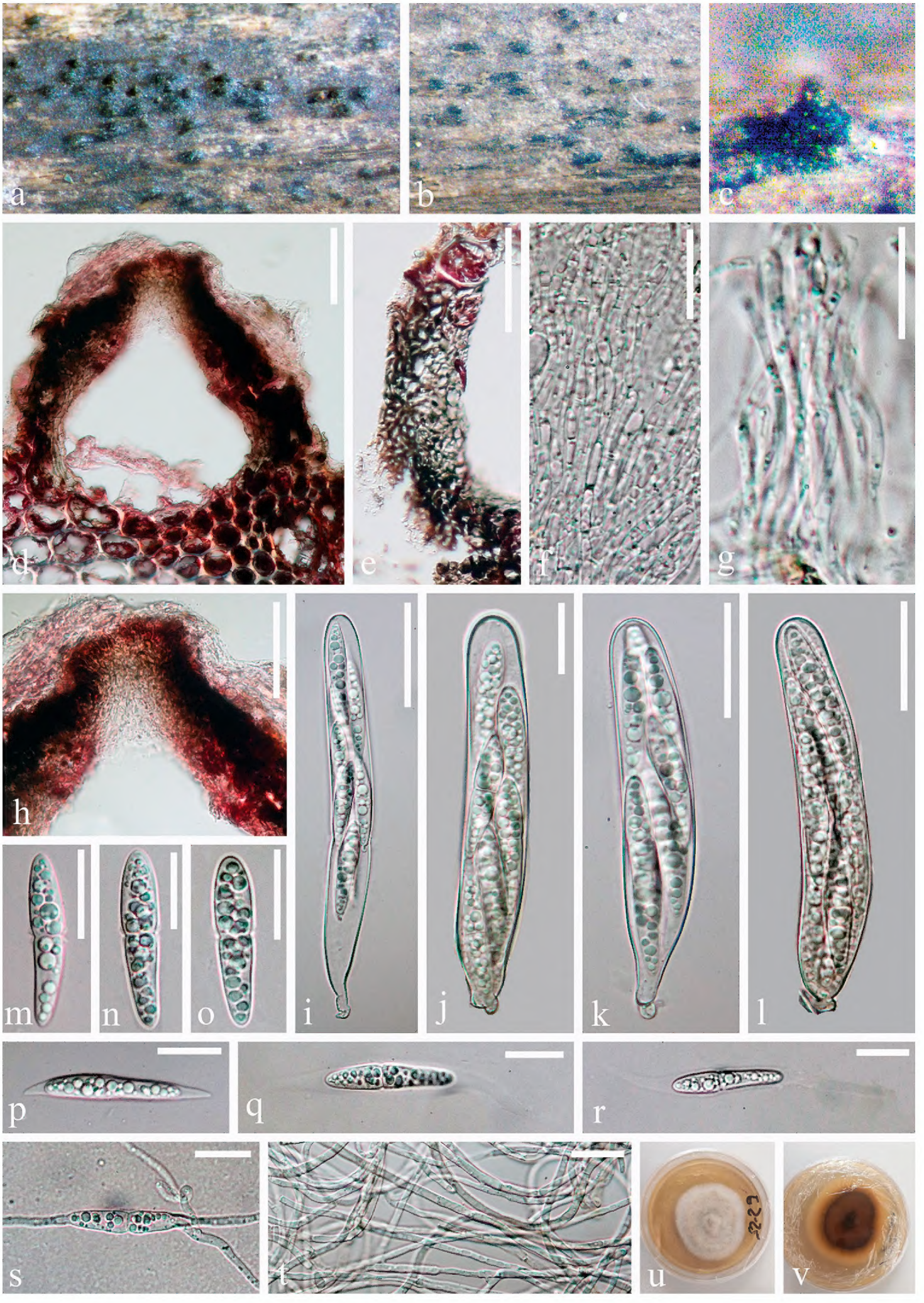

Figure 2. *Tingoldiago hydei* (MFLU 19–2842, holotype). **a–c** Ascomata on wood **d** section of ascoma **e** peridium **f, g** pseudoparaphyses **h** ostiole **i–l** asci **m–r** ascospores **s** germinating ascospore **t** vegetative hyphae in culture **u, v** culture on PDA from surface and reverse. Scale bars: 50 μm (**d, e, h**), 20 μm (**f–g, m–t**), 30 μm (**i–l**).

However, *T. clavata* differs from *T. raminicola* in having longer asci (95–164 × 18–22 vs. 87.5–122 × 18.25–25 μm) and smaller ascospores (37.5–42 × 7.5–9 vs. 43.5–53 × 7.5–11 μm). Moreover, ascopores of *T. clavata* have short appendages at the septum while ascospores of *T. raminicola* lack appendages. In addition, we compared the base pairs of ITS regions between these two species and there were 25 base pairs without gaps (5.1%) differences. Therefore, we introduce our isolate as a new species based on both phylogeny and morphological characters.

Tingoldiago clavata **D.F. Bao, L. Xu & H.Y. Su, sp. nov.**
Index Fungorum No: IF557048
Facesoffungi No: FoF07083
Figure 3

Etymology. Referring to the clavate ascospores of this fungus.

Holotype. Thailand, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B-161 (MFLU 19–2843, holotype), ex-type culture, MFLUCC 19–0496.

Description. *Saprobic* on submerged decaying wood. ***Sexual morph*:** *Ascomata* 145–210 × 145–195 μm ($\overline{x}$ = 175 × 169 μm, n = 10), immersed to semi-immersed, gregarious, scattered, erumpentia, depressed globose to conical with a flattened base, dark brown to black, as dark spots on host surface. *Ostiole* central, round to papillate, short, crest-like, dark brown. *Peridium* 28–47 μm wide, comprising several layers, pale brown to brown cells of *textura anngularis*. *Hamathecium* comprising 1.5–2.0 μm (n = 30) wide, numerous, branched, septate, hyaline, cellular pseudoparaphyses. *Asci* 110–148 × 20–27 μm ($\overline{x}$ = 129 × 23 μm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical-clavate, rounded at apex, with a short pedicellate. *Ascospores* 48–51 × 7.5–9 μm ($\overline{x}$ = 50.5 × 8.5 μm, n = 30), overlapping, 2–3-seriate, clavate, with round ends, straight, uniseptate, deeply constricted at septum, hyaline, with broad and short upper cells 16.6–18.9 × 7.8–9.0 μm ($\overline{x}$ = 17.7 × 8.4 μm, n = 30), narrow and long lower cells 30–32.9 × 6.5–8.0 μm ($\overline{x}$ = 31.5 × 7.3 μm, n = 30), tapering towards the end, guttulate, smooth, 2–4 equatorial appendages at the septum and surrounded by a fusiform gelatinous, sheath. ***Asexual morph*:** Undetermined.

Culture characteristics. Ascospores germinating on PDA within 24 hours. Colonies on MEA effuse, velvety, greyish-white to dark brown from above and below, reaching 2.5–3 cm diameter within 30 days at room temperature under natural light, composed of subhyaline to brown, septate, smooth hyphae.

Additional specimens examined. Thailand, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B160 (paratype: MFLU 19–2844; living culture, MFLUCC 19–0498); Thailand, That Phanom, Nakhon Phanom, on submerged decaying wood, 13 November 2018, D.F. Bao, B136 (paratype: MFLU 19–2845; living culture, MFLUCC 19–0495)

Figure 3. *Tingoldiago clavata* (MFLU 19–2843, holotype). **a–c** ascomata on wood **d** section of ascoma **e** ostiole **f** peridium **g** pseudoparaphyses **h–l** asci **m–r** ascospores **s** vegetative hyphae in culture **t, u** culture on PDA from surface and reverse. Scale bars: 50 μm (**d, e**), 20 μm (**f–l**), 10 μm (**m–s**).

Notes. *Tingoldiago clavata* resembles the type species, *T. graminicola* in having bitunicate, cylindrical-clavate asci with a short pedicellate and clavate, hyaline, 1-septate, ascospores with broad upper cells, narrow lower cells. However, we can distinguish them by the size of ascomata and asci and the colour, septate and appendages of ascospores. *Tingoldiago clavata* has smaller ascomata (110–148 ×145–195 vs. 150–250 × 250–450 μm) and larger asci (110–148 × 20–27 vs. 87.5–122 × 18.25–25 μm). Moreover, ascopsores of *T. clavata* are hyaline, uniseptate, with 2–4 equatorial appendages at the septum, while ascopspores of *T. graminicola* are brown and 3-septate at maturity and lacking appendages at the septum. In addition, a comparison of the 491 nucleotides across the ITS gene region of *T. clavata* and *T. graminicola* reveals 25 base-pair differences and therefore provides further evidence to introduce *T. clavata* as a new species as recommended by Jeewon and Hyde (2016).

Discussion

During the last decade, freshwater fungi in Thailand have been mainly reported from north, south and northeast of Thailand (Jones et al. 1999, Marvanová and Hywel-Jones 2000, Sivichai and Boonyuen 2010, Sivichai and Hywel-Jones 1999, Sivichai et al. 1998, 2000, Sri-indrasutdhi et al. 2010). No freshwater fungi from Eastern Thailand have been reported so far. In this study, two new freshwater species, viz. *Tingoldiago hydei* and *T. clavata* from Eastern Thailand, are introduced, based on morphology and phylogeny. *Tingoldiago hydei* and *T. clavata* satisfied the generic concept of the genus *Tingoldiago* (Hirayama et al. 2010). They comprise globose to conical, immersed to erumpent ascomata, cellular pseudoparaphyses, bitunicate, fissitunicate asci and clavate ascospores with a median primary septum and a large fusiform gelatinous sheath around the ascospore (Hirayama et al. 2010). Morphologically, *T. hydei* and *T. clavata* are quite similar as they have similar shape of asci and ascospores; however, we can distinguish them by the size of ascomata, asci and ascospores (Table 2). In addition, we also compared the morphological differences of these two species with the type species, *T. graminicola*. Ascopores of *T. hydei* and *T. clavata* are hyaline, uniseptate, with appendages at the septum and the upper cells are broader and shorter than the lower cells, while the ascopsores of *T. graminicola* are hyaline, uniseptate, but becoming brown and 3-septate with age, lacking appendages at the septum, upper cells and lower cells are similar lengths. Phylogenetic analyses showed that our two new isolates clustered together and are sister to the type species, *Tingoldiago graminicola* with strong bootstrap support (100 ML/92 MP/1.00 PP). This evidence strongly supports our two isolates to be the new species.

Hyde et al. (2020) introduced a new genus, *Pseudomurilentithecium* in Lentitheciaceae. In their phylogenetic analysis, *Pseudomurilentithecium* clustered with *Poaceascoma* and was basal to Lentitheciaceae. However, in our phylogenetic analysis, *Pseudomurilentithecium* grouped with the members of Massarinaceae, rather than Lentitheciaceae. Therefore, further investigation is required to confirm the placement of the genus.

Table 2. The morphological comparisons of *Tingoldiago* species discussed in this study.

Taxa	Distribution	Ascomata (µm)	Pseudoparaphyses (µm)	Asci (µm)	Ascospores (µm)	References
Tingoldiago graminicola	Japan, UK	150–250 × 250–450	1.5–4	87.5–122 × 18.25–25	43.5–53 × 7.5–11	Hirayama et al. 2010
T. hydei	Thailand	180–280 × 330–470	1.8–2.5	95–164 × 18–22	37.5–42 × 7.5–9	This study
T. clavata	Thailand	145–210 × 145–195	1.4–2.0	110–148 × 20–27	48–51 × 7.5–8.5	This study

Tingoldiago is a well-resolved genus in this family with a stable clade within Lentitheciaceae. The genus can be distinguished from other genera in this family by having hyaline, uniseptate, upper cells are broad and basal cells are narrow ascospores with a large fusiform gelatinous sheath. The sheath is considered to be an adaptation by the genus that enables ascospores to attach to the substrates in moving water (Shearer 1993, Hyde and Goh 2003, Jones 2006, Devadatha et al. 2019). It is reported that the genus *Tingoldiago* is exclusively found in freshwater habitats (Hirayama et al. 2010) and our two new species were collected from lotic habitats of Mekong River.

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